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ORIGINAL ARTICLE

Evaluation of copper speciation in the extract of *Eichhornia crassipes* using reverse and forward/CLE voltammetric titrations

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Abstract Reverse and forward titrations coupled with the competitive ligand equilibration (CLE) technique were, for the first time, used to investigate the chemical organic speciation of copper ions in an aqueous extract of *Eichhornia crassipes* (*E.C*) plant growing in Egypt. Salicylaldoxime (SA) was used as the competitive ligand. Two samples were collected from huge submerged masses in the Nile water at Sohag and Mansura cities. In this study the concentration of the natural complexing ligands (C_{Ln}) and their conditional stability constants with copper (K'_{CuLn}) in the aqueous extract were determined by linear and nonlinear modeling. Reverse titration resulted in two classes of copper complexation (CuL1 and CuL2) in both samples. The strongest class, CuL1, is predominant in Sohag-sample with average $\log K'_{CuL1}$ and C_{L1} of 15.66 and 435.5 nM, respectively, and also dominated in the Mansura-sample with average 16.77 and 162 nM, respectively. The second class (CuL2) is minor in both samples. For comparison, forward titration was performed for the Sohag-sample. The results indicated that reverse titration showed a breakthrough ability to detect, for the first time, different types of metal complexation present in equilibrium in the aqueous extracts of biological samples. Moreover, the presence of *E.C* in Nile water is so useful in transforming free Cu ions to less toxic forms by organic complexation.

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1. Introduction

Eichhornia crassipes (Mart.) Solms (*E.C*) is a native Brazilian plant but has been naturalized in many tropical/subtropical countries (e.g. Egypt). It is known as a nuisance because of its fast growth rate. Although *E.C* is considered as an invasive hyacinth, it could be useful as a source of biomass, because it is abundant and easy to cultivate. A previous work has discussed the potential use of *E.C* as a biomass, a way to improve water quality because of its capacity to absorb heavy metals and

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organic compounds and a source of ethanol (Masami et al., 2008).

It is important to discuss the benefits as well as negative effects of *E.C* towards the aquatic environment. Later, much attention was given to the use of *E.C* as a biosorbent to remove heavy metals from waste water, because it has high affinity to eliminate metal ions. A few manuscripts revealed that the *E.C* causes serious problems in nearly all countries, affecting almost all uses of water bodies such as aquaculture, commercial and subsistence fishing, drinking and household consumption, hydropower generation, irrigation, transport and recreation (Villamagna and Murphy, 2010; Charudattan, 2001). *E.C* has variable chemical contents which occurred through functional groups that are native to protein, lipids, and carbohydrates. Although *E.C* has been used as a biosorbent for many toxic metal ions (Schneider et al., 1995), no studies were done to evaluate different chemical speciation of metal ions in its aqueous extract. To evaluate this study, copper, in natural sample, was selected for the following reasons: (i) it is highly toxic to human and river organisms (ii) it is strongly complexed by organic chelators in natural water, which lowers its toxicity (Moffett et al., 1997; Donat et al., 1994).

Sea and River waters are the most abundant and important natural fluid, in which a number of physical and chemical processes of environmental and biological relevance occurs. For instance, the natural degradation of the aquatic plants like *E.C* produces, after death, different organic molecules which in turn dissolve in the water habitat and react with metals to form heterogeneous mixture of complexes. To gain a thorough understanding of all the processes occurring in the water habitat, speciation studies are of fundamental importance, especially if one takes into account the complexity of these water systems, a multielectrolyte aqueous solution in which a wide number of cations and ligands are present. For example, a correct study of the distribution of an anionic component in its different species theoretically needs the consideration of all its possible interactions with all cations (at least) present in water, making this task very challenging (for a comprehensive discussion on these topics one may refer, e.g., to some important papers and books) (Crea et al., 2008).

For environmental reasons, determination of the bioavailable fraction of trace metals has gained more attention than the total metal determination since the total metal concentration provides a little information about its bioavailability because: (i) the total concentration of a metal can vary significantly from one aquatic system to another due to the variations in the chemical forms of the metal ions, (ii) the total concentration of elements cannot provide the required information about mobility and the impact of elements on ecological systems or biological organisms, as elements usually interact as parts of macromolecules (proteins, enzymes, etc.) or according to their oxidation state, (iii) the quality and quantity of the prospective element species in a matrix are highly responsible for the mobility, bioavailability and the ecotoxicological or toxicological impact of the element rather than the total element concentration (Templeton et al., 2000). (iv) with increasing knowledge of the environmental consideration, metabolism and biological effects of trace elements has become increasingly apparent that there is a need not only to determine the total levels of an element in water or soil, tissues and body fluids but also to measure its different chemical forms (speciation) quantitatively.

Numerous methods have been developed to determine copper speciation in aquatic systems directly or indirectly (ion selective electrodes, voltammetric methods and ion exchange technique) (Xue and Sunda, 1997; Zhang and Davison, 1995). Previously, the concentration of copper complexing ligands and the stability of the complexes were determined by anodic stripping voltammetry (ASV) (Ndungu et al., 2005) or by Cathodic Stripping Voltammetry (CSV) with ligand competition (van den Berg, 1985). A more promising technique that has been developed over the last decade is the Adsorptive Differential Pulse Cathodic Stripping Voltammetry (AdCSV), which has achieved great success resulting from its low limits of detection and general applicability to many more elements than DPASV. Recently, the AdCSV technique with an added organic reagent as a competitive ligand (CLE-AdCSV) was preferred for studying the speciation of trace elements. This is due to the sensitivity, versatility and simplicity of this technique. The organic ligand in natural water participating in competition of Cu(II) fall in at least two major groups which include stronger ligand (L1) and weaker ligand (L2) (Coale and Bruland, 1988). It is important to mention that no information is available about the chemical nature and structure of ligands on the aquatic extract of *E.C*. Moreover, reverse and forward titrations were never applied before on biological aqueous extracts of plants but were only applied before to study the chemical speciation in the river and sea waters.

This study aims to apply the reverse/forward voltammetric titrations with CLE technique to determine the chemical speciation of Cu in the aqueous extract of two *E.C* samples. The chemical speciations of Cu were evaluated by determining the concentration of the natural complexing ligands, [Ln], and the conditional stability constant, K'_{CuLn}, of Cu–L system. To achieve this work experimentally, reverse and forward titrations were performed by AdCSV/CLE technique using SA as a competing ligand. Data evaluation was made by van den Berg and Scatchard Linearizations for the forward titration as well as nonlinear models for the reverse titration.

2. Materials and methods

2.1. Chemical reagents, preparation of solutions and apparatuses

All chemical reagents were of Analytical grade from Merck and BDH. A stock solution of copper (1000 mg L^{-1}) was prepared from $Cu(NO_3)_2 \bullet 3H_2O$ in ultrapure water. A stock solution of 0.1 M SA ligand was prepared in 0.1 M of HCl. A 1.0 M boric acid was mixed in 0.35 M ammonia to prepare 1.0 M of borate buffer. The borate buffer was purified by soaking with MnO_2 and UV irradiation (~2 h for each step) to eliminate any copper ions and organic substances, respectively. All solutions were kept in a refrigerator at 5 °C until measurements were undertaken.

EG&G Princeton, voltammetric system model 264A, equipped with a 303A hanging mercury drop electrode (HDME) was used in the voltammetric titrations. A three-electrode combination, consisting of a working electrode (HMDE), a saturated Ag/AgCl reference electrode and a Pt wire auxiliary electrode was used. An EG&G *X–Y* recorder model RE 0089 from Princeton was used to obtain the voltammograms.

Orion digital pH–mV meter model 701A was used to estimate the pH value in aqueous extract. A Barnstead E-pure

ultrapure water purification system was used. All glassware were cleaned by soaking in 1:1 HNO_3, HCl and one weak in ultrapure water. More description of this method is available elsewhere (Komy, 1993).

2.2. Sampling and preparation of E.C for speciation measurements

Leaves and stems from *E.C* were collected from large floating masses in the River Nile at Sohag city (~500 Km southeast Cairo) and at Mansura city (~120 Km Northeast Cairo), Egypt. These samples were washed with Nile, tap and ultrapure waters to remove any sand and other debris, freeze dried, grounded and sieved through 0.25 mm mesh sieve.

An aqueous extract of *E.C* was prepared by transferring 1.0 g dry weight of biomass into a 500 mL flask containing 250 mL ultrapure water, shaking (120 min) and centrifuging (15 min) the suspension to separate the solid phase. The extract was then transferred to 1.0 L volumetric flask and filled to the mark using ultrapure water.

2.3. Total dissolved copper $[Cu]_T$ by AdCSV in E.C extract

Total dissolved copper in the acid-digested aqueous extract of *E.C* samples was determined by AdCSV technique (Campos and van den Berg, 1994) as follows; 10 mL of acid-digested solution was mixed with 100 μL of SA (0.1 M) and 150 μL borate buffer (pH 8) into a preconditioned voltammetric cell. The solution was then deaerated with N_2 gas for 5 min then left to equilibrate for 10 min.

The scan parameters were deposition potential of −0.15 V; deposition time of 2.0 min to a fresh mercury drop while stirring; a quiescence period of 15 s; AdCSV scan in a negative-going differential pulse technique from −0.15 to −0.63 V with 10 mV s^{-1} scan rate; 50 mV pulse amplitude. Measurements were repeated after standard copper addition (from 0.948 to 2.844 μM) to calibrate the sensitivity.

2.4. pH and time optimization for reverse/forward titrations

The optimum pH for Cu-SA complex formation was determined as follows; 1.0 mL from *E.C* extract was mixed with 60 μL SA (0.1 M) at different pH's (7.5–8.31) and completed to a total volume of 10 mL using ultrapure water. The cells were left to equilibrate for 5.0 h. The scan parameters were the same as in Section 2.3.

To illustrate the influence of equilibration time on Cu-SA system; 1.0 mL extract was mixed with 60 μL SA (0.1 M) and 150 μL borate buffer (pH 8.0) into clean quartz electrochemical cell and completed to 10 mL with ultrapure water the left to equilibrate at different times (10–450 min). The scan parameters were the same as in Section 2.3.

2.5. Reverse and forward titrations of copper complexing ligands

Reverse titration was applied as before (Nuester and van den Berg, 2005); a 9 mL portion *E.C* extract was transferred to a voltammetric cell, and 150 μL of borate buffer was added. The concentration of SA was increased stepwise to give concentration up to 0.3 mM (in Sohag-sample) and 0.4 mM (in Mansura-sample) and complete to a final volume of 10 mL using ultrapure water. The solutions were left for 5 h in dark at 25 °C to attain equilibrium. Record the AdCSV voltammograms using the same parameters used in Section 2.3.

Forward titration was performed as described before (Nuester and van den Berg, 2005); about 100 mL of *E.C* extract was transferred to a pre-cleaned PTFE bottle, borate buffer was added to a concentration of 0.01 M, and 0.98 mM SA. In a set of 10 polystyrene tubes, transfer 10 mL of this mixture at varied copper concentration between 0 and 20 nM. The samples were equilibrated for 17 h/overnight in dark at 25 °C. The samples were transferred to the voltammetric cell, and the concentration of the Cu-SA complex was determined using AdCSV as before. The same experiment was repeated with a UV-digested aqueous extract (for 2 h) to calculate the sensitivity (S).

3. Results and discussion

3.1. pH and time optimization for reverse/forward titrations

3.1.1. pH optimization

Fig. 1a shows the effect of pH on i_p of Cu-SA complex. Two significant peaks at −0.25 and −0.4 V are shown in Fig. 1a, corresponding to free SA and Cu-SA complex respectively. It can be seen from the figure that i_p at −0.4 V gradually increases with increasing pH to 7.88. Afterwards, it maintains constant till pH 8.05. Further increase in pH leads to rapid

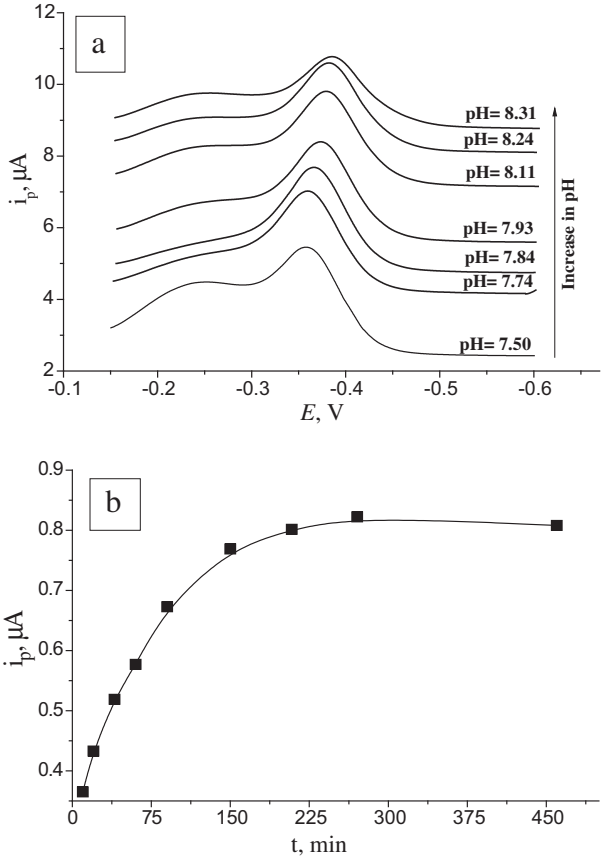

Figure 1 Effect of pH and time on Cu-SA electrochemical signal: (a) voltammograms of Cu-SA system at pH (7.5–8.31) and (b) plot of peak current of Cu-SA complex (i_p) *vs.* time using AdCSV technique.

decrease in the i_p. The maximum current ($i_{p(max)}$ = 1.67 μA) was reached at pH 7.98.

Also, it can be noticed that the peak potential of Cu-SA complex shifts towards a more negative potential with increasing pH, about 0.027 V/pH units during the pH range 7.93–8.24. Both the increase in i_p and the negative-shift in peak potential are due to the increase in Cu-SA stability. The decrease in the peak current at pH > 8.05 is attributed to the hydrolysis of copper. Therefore, pH 8.0 was selected as the optimum pH for the forward/reverse titrations. This result is similar to that in a previous study by Jin and Gogan, (2000). The above results reflect the following: (i) peak potential at −0.25 V is related to the free SA which decreases with copper addition and (ii) stability of copper-SA complex occur until pH 8.1.

3.1.2. Time optimization

Fig. 1b illustrates the influence of equilibrium time on Cu-SA signal (i_p). Clearly, the i_p grows rapidly in the period 10–150 min. After then, the i_p increases slowly up to 5 h. Any further extension in the equilibrium time leads to insignificant change in the i_p. So that, the optimum time for Cu-SA complexation in the extract is 5 h. A similar result was obtained by Campos et al. through their study on Cu speciation in sea water (Campos and van den Berg, 1994).

3.2. Characteristics of E.C sample

Analysis of amino acid showed that *E.C* extract contains 16 amino (14 aliphatic and 2 aromatic) acids. The scores of amino acids are 9.28, 1.15, 0.86, 0.64, 0.55, 0.47, 0.39, 0.38, 0.35, 0.33, 0.32, 0.31, 0.30, 0.28, 0.27, 0.11 mg g^{-1} for proline, glutamic acid, aspartic acid, leucine, alanine, lysine, arginine, serine, glycine, phenylalanine, tyrosine, thereonine, valine, histidine, isoleucine and methionine, respectively. In other words, proline, glutamic and aspartic acids are the major amino acids in the extract (70.6%), while histidine, isoleucine and methionine are minor (4.12%). These findings suggest that *E.C* is a good source of complexing ligands with affinity to chelate with metal ions, e.g. Cu^{2+}.

Energy dispersive X-ray (EDAX) was used to analyze the *E.C* elemental content in its powder form. The analysis revealed that Cu represents about 0.7% of the *E.C* elemental content. This is attributed to the biosorption/chelation nature of *E.C* to different metals existing in the Nile water where the plant long-lived.

Analysis of major cations and anions in the *E.C* extract is so important in calculating the side reaction coefficient of SA with them. The results showed that (Na^+, K^+, Ca^{2+} and Mg^{2+}) and (Cl^-, HCO_3^- and SO_4^{2-}) are predominant in the extract recording the concentrations (19.8, 656, 23.5 and 20.04 mg L^{-1}) and (727, 576 and 22 mg L^{-1}), respectively. In other words, contents of cations are in the order $K^+ > Ca^{2+} > Mg^{2+} > Na^+$ while anions in the order $Cl^- > HCO_3^- > SO_4^{2-}$.

3.3. Total copper $[Cu]_T$

Using standard addition method, the average value of $[Cu]_T$ after three additions of standard Cu was found 0.48 μM in Sohag-sample and 0.144 μM in Mansura-sample. These values will be used for further calculations in both reverse and forward titrations. To estimate the accuracy of this method, an artificial sample of Cu(II) was analyzed in the same manner. Briefly, standard Cu^{2+} was added into a clean voltammetric cell containing 1.5 mM SA to have a final concentration of Cu^{2+} = 1.52 nM (artificial sample). The pH was adjusted at 8.0 using borate buffer. Three standard additions of Cu^{2+} of known concentrations were used to detect the concentration of copper in the artificial sample under the optimum condition (Campos and van den Berg, 1994). The average value (±SD) of Cu^{2+} in the artificial sample was 1.46 nM (±0.1). The relative error was found 3.95%. Thus, proposed procedure is accurate to detect the total Cu^{2+} in the acid-digested extract.

Clearly, the level of Cu in Sohag-sample is 3.3 times that in Mansura-sample. This reflects the difference in plant affinity to adsorb Cu due to different habitat.

3.4. Reverse and forward titration of copper complexing ligand

River Nile is characterized with high population of *E.C* aquatic plant. Since no data have been published on the natural speciation of Cu in the aqueous extract of *E.C*, our results are compared with previous data of sea and estuarine waters (Campos and van den Berg, 1994; Nuester and van den Berg, 2005; Apte et al., 1990; Santos-Echeandía et al., 2008). Leal et al. (1999) indicated that copper complexing ligands in sea water are known to be produced by marine fungi and cyano bacteria with strong complexing stability.

Recently, concentration of copper complexing ligand [Ln] and conditional stability constants (K'_{CuLn}) were determined by competitive ligand equilibration/adsorptive differential pulse Cathodic Stripping Voltammetry (CLE/AdCSV) using reverse and forward titrations (van den Berg, 1985; Nuester and van den Berg, 2005). Generally, CLE/AdCSV titration is based on the competition between the natural ligands (Ln) and the added ligand (SA) to form complexes with Cu.

3.4.1. Reverse titration

The reverse titration is a new method to detect complexing ligands and their stability constants with copper metal ions in aqueous extract of *E.C*. It is based on changing the concentration of a competing ligand (C_{SA}) from low to high with detecting the analytical response (i_p) related to (Cu-SA) complex using AdCSV technique while keeping the copper metal ion concentration constant in the titration system (Nuester and van den Berg, 2005). The peaks current (i_p), AdCSV response, is directly related to the concentration of the electroactive and adsorptive species (Cu-SA). So, different copper species can be determined by measuring the i_p during titrations with SA. The relative current response ($i_p/i_{p\bullet max}$) is required to determine speciation parameters. An advantage of the proposed procedure that is necessary to obtain the value for $i_{p\bullet max}$ which is the maximum current that is approached at high SA concentration (Nuester and van den Berg, 2005). The $i_{p\bullet max}$ can be determined by plotting i_p (Y-axis) against [SA] (X-axis), as shown in Fig. 2. Clearly, the AdCSV response (i_p) grows rapidly from 0.1 to 0.58 μA with increasing C_{SA} from 0.0 to 0.085 mM. After then, the i_p rises slowly from 0.58 to 0.68 μA on increasing [SA] from 0.058 to 0.15 mM. The increase in i_p during these two regions is attributed to the progress in Cu chelation with SA to produce the electroactive

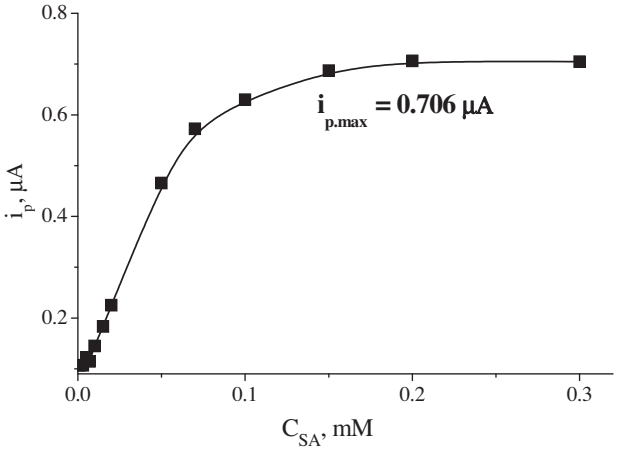

Figure 2 Reverse titration; a plot of analytical response (i_p) obtained from the voltammograms *vs.* C_{SA}, showing an $i_{p\bullet max}$ of 0.706 µA at 0.2 mM SA.

species (Cu-SA). Any further increase in C_{SA} (>0.15 mM) results in constant i_p resonse (0.706 µA). At this point, the peak height is maximal $i_{p\bullet max}$. Campos and van den Berg (1994) demonstrated that there are two Cu^{2+}/SA complexes, $CuSA_1$ and/or $CuSA_2$, formed at the maximum current ($i_{p\bullet max}$). These two forms of Cu-SA complexation can adsorb at the electrode, but they considered that the $CuSA_2$ species are the predominant so, they are more likely adsorbed. This observation, also, was confirmed by Nuester and van den Berg (2005).

As $i_{p\bullet max}$ is directly related to concentration of the electroactive/adsorptive species, the AdCSV response can be modeled by calculation of the abundance of this species during titration with relative current response. Therefore, the quantitative description of the relative current response ($i_p/i_{p\bullet max}$) requires a theoretical model for determining the different ligands concentration, C_{Ln}, and their conditional stability constants, K'_{CuLn}, in the extract. Accordingly, relative current response ($i_p/i_{p\bullet max}$) was modeled as function of $-\log C_{SA}$. So, $i_p/i_{p\bullet max}$ was fitted along with the parameters (C_{Ln} and K'_{CuLn}) using the following the equations:

For one ligand modeling;

$$X_{theo} = \frac{i_p}{i_{p\cdot max}} = \frac{\beta_{CuSA_2}(C_{SA})^2}{\alpha_{Cu(inorg)} + K_{CuSA}C_{SA} + \beta_{CuSA_2}(C_{SA})^2 + \frac{K'_{CuL_1}C_{L_1}}{1+K'_{CuL_1}C_{L_1}}} \quad (1)$$

For two ligands modeling;

$$X_{theo} = \frac{i_p}{i_{p\cdot max}} = \frac{\beta_{CuSA_2}(C_{SA})^2}{\alpha_{Cu(inorg)} + K_{CuSA}C_{SA} + \beta_{CuSA_2}(C_{SA})^2 + \frac{K'_{CuL_1}C_{L_1}}{1+K'_{CuL_1}[Cu^{2+}]} + \frac{K'_{CuL_2}C_{L_2}}{1+K'_{CuL_2}[Cu^{2+}]}} \quad (2)$$

where X, $\alpha_{Cu(inorg)}$, C_{SA}, $[Cu^{2+}]$, K_{CuSA} and β_{CuSA_2} represent the actual over the maximum peak current ($i_p/i_{p\bullet max}$) at each titration point, the inorganic side reaction coefficient of Cu, added ligand (SA) concentration, free Cu^{2+} concentration, conditional stability constant of $CuSA_1$ complex type and conditional stability constant of $CuSA_2$ complex type, respectively. Also, K'_{CuL1}, K'_{CuL2}, C_{L1} and C_{L2} represent stability constant of natural complex (CuL1), stability constant of natural complex (CuL2), concentration of natural ligands (L_1) and concentration of natural ligands (L_2).

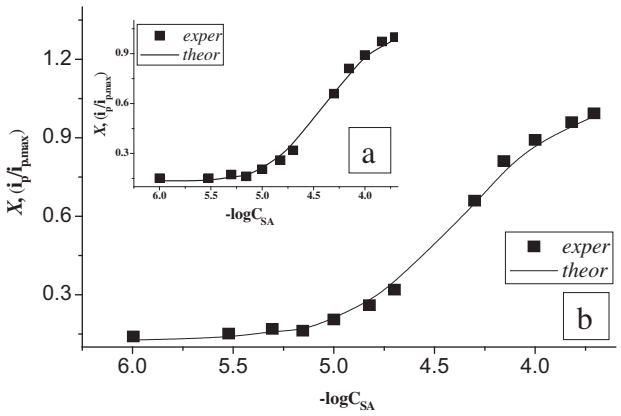

Figure 3 NLLSR fitting plot of reverse titration for Sohag-sample to: (a) one ligand and (b) two ligands assumptions.

To calculate the values of C_{L1}, C_{L2}, K'_{CuL1} and K'_{CuL2} in Eqs. (1) and (2), Non-Linear Least Squares Regression method (NLLSR) was applied as before (Nuester and van den Berg, 2005).

Figs. 3 and 5a and b show the NLLSR fitting plot of the reverse titration for Sohag- and Mansura-sample, respectively. Clearly, the theoretical data (continuous line) in Figs. 3 and 4a and b fit well with experimental data (dotted line). Tables 1 and 2 summarize the values of speciation parameters as well as the correlation coefficient (R^2) for Sohag- and Mansura-samples, respectively. Although there have been outstanding fitness between the experimental and theoretical lines for both samples using Eqs. (1) and (2), but Eq. (2) is more applicable as its values of R^2 (0.999) are the highest for both samples. Namely, the hypothesis of the presence of two classes of ligands, L_1 and L_2 is more reliable. It is clear from the Tables 1 and 2 that the value of C_{L1} in Sohag-sample is about 3.5

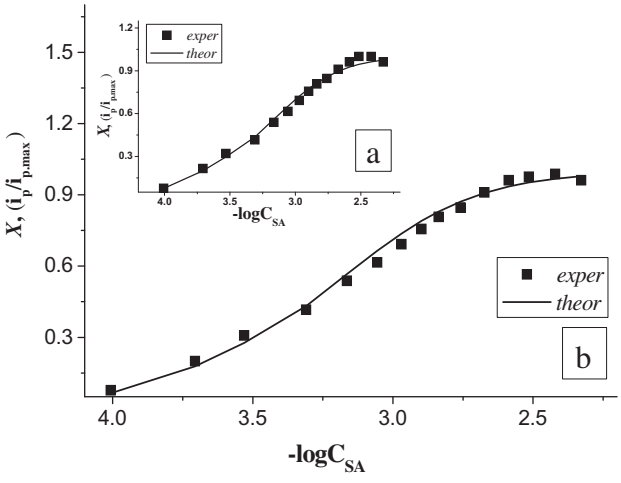

Figure 4 NLLSR fitting plot of reverse titration for Mansura-sample to: (a) one ligand and (b) two ligands assumptions.

Table 1 Values of conditional stability constants and natural ligands concentrations in the aqueous extract of *E.C* sample collected from Sohag city using reverse and forward titrations.

AdCSV			Speciation parameters					
Type of titration	Technique		$\log K'_{CuL1}$	$\log K'_{CuL2}$	C_{L1} (nM)	C_{L2} (nM)	[a]C_{LT} (nM)	R^2
Reverse titration	NLLSR	-one ligand	13.35 ± 0.21	–	441 ± 6.1	–	441	0.995
		-two ligands	17.96 ± 0.33	10.7 ± 0.22	430 ± 4.3	103 ± 2.1	533	0.999
Forward titration	van den Berg linearization		18.56 ± 0.14	–	473 ± 5.0	–	473	0.998
	Scatchard linearization		18.06 ± 0.35	–	533 ± 3.7	–	533	0.956

[a] $C_{LT} = C_{L1} + C_{L2}$.

Table 2 Values of conditional stability constants and natural ligands concentrations in the aqueous extract of *E.C* sample collected from Mansura City.

AdCSV			Speciation parameters					
Type of titration	Technique		$\log K'_{CuL1}$	$\log K'_{CuL2}$	C_{L1} (nM)	C_{L2} (nM)	C_{LT} (nM)	R^2
Reverse titration	NLLSR	-one ligand	16.96 ± 0.43	–	122 ± 3.1	–	122	0.991
		-two ligands	16.77 ± 0.22	13.23 ± 0.27	162 ± 3.9	1.0 ± 0.01	163	0.999

times in Mansura-sample. Whereas, C_{L2} of Sohag-sample is about 100 times that of Mansura-sample. The values of $\log K'_{CuL1}$ calculated from Eqs. (1) and (2) for both samples are almost similar (just above/below 17.0), with the exception of that calculated by Eq. (1) for the Sohag-sample which have much lower value ($\log K'_{CuL1} = 13.35$). Finally, $\log K'_{CuL2}$ of Mansura-sample (13.23) is higher than of Sohag-sample (10.76). These variations in the complexing ligands concentration and their conditional stability constants are related to the variation in the living conditions of the plants, where both nutrients and weather conditions are different in Sohag and Mansura cities. Previous studies by Santos-Echeandía et al. (2008) indicated that the above calculation with the two models (one- and two-ligands models) are inaccurate at ligand concentrations much greater than the copper concentration, which means that the method of reverse titrations is most suitable for ligands with concentrations similar to that of the metal. Actually, the total ligand concentrations, C_{LT}, detected by the reverse titrations are similar to the $[Cu]_T$ in both *E.C* samples, i.e. the ligand concentrations were low.

The values of $\log K'_{CuL1}$, determined by reverse titration in the present study, are very high with an average of 15.66 and 16.87 for Sohag- and Mansura- samples, respectively, Tables 1 and 2. Town and van Leeuwen (2005) suggested that the kinetics of very stable complexes should be slow. So, the equilibrium between the natural complexes and the added ligands could not be obtained. This assumes that the natural complex needs to dissociate prior to the formation of the complex with the added ligand (SA), and that the reaction follows the Eigen mechanism. It means that the rate of formation of the natural complex (CuL_n) is limited by the loss of water from the inner coordination sphere of the metal. This observation indicates that the upper limit of $\log K'_{CuL1}$ is ~14.0 in the water samples for overnight equilibrium, with an uncertainty of about a unit in $\log K'_{CuL}$ (Morel and Hering, 1993). However, the average values of $\log K'_{CuL1}$, determined by the reverse titration in the two *E.C* samples (15.66 and 16.87), are not compatible with the Eigen mechanism. Nagai et al. (2007) discussed before the shortfalls of Eigen mechanism. Moreover the same author concluded that, if the complexation reaction happens with an adjunctive pathway (Morel and Hering, 1993), the theory would not be valid as the outer-sphere complex is not produced. The present results indicate that the exchanging reaction between L_n and SA in the *E.C* extract obey the adjunctive route and reach the best equilibrium within about 5 h for the reverse titration. Whilst, previous studies showed that the equilibrium in the reverse titration is reached in 30 min (Nuester and van den Berg, 2005) and in 1 h (Santos-Echeandía et al., 2008).

Previously, Santos-Echeandía et al. (2008) and Laglera and van den Berg (2006) indicated that the conditional stability constant $\log K'$ of Cu complexes with the natural ligands, found in Vigo Ria estuary ($\log K'_{CuL1} \sim 13.2$) (Santos-Echeandía et al., 2008) and in Scheldt estuary ($\log K'_{CuThiol} \sim 13$) (Laglera and van den Berg, 2006), correspond to thiol compounds. This result conforms to our result of $\log K'_{CuL1}$ (13.35), calculated with one ligand assumption for Sohag-sample, and also similar to $\log K'_{CuL2}$ (13.23), calculated with two ligands assumption for Mansura-sample, Table 2. This finding suggests that some of the complexing ligands in *E.C* extract are from thiols compounds and this is an expected result, where the *E.C* extract is enriched with methionine and cysteine amino acids. Refer to the results of plant characteristics in Section 3.2.

3.4.2. Forward titration

Forward titrations/competitive ligand equilibration (CLE) technique is based on starting the titration with a fixed concentration of SA, below the original free copper ion concentration; the copper metal is distributed over the added (SA) and the original ligands (L_n). During the titration with copper, the strong species are formed first, followed by the weaker species. For this reason, titrations should be carried out at a high detection window and at a realistic range of metal concentration. In order to quantify the Natural ligands and their complexing stability constants with copper in the *E.C* extract,

forward titration was applied only for the Sohag-sample for comparison. The forward titrations of copper-ligand complexes were performed as before (Campos and van den Berg, 1994), by using linearization by and Scatchard (1949) as follows:van den Berg equation;

$$[Cu]_{labile}/[CuL_n] = [Cu]_{labile}/C_{Ln} + \alpha'/K'_{CuLn}C_{Ln} \quad (3)$$

Scatchard equation;

$$[CuL_n]/[Cu]_{labile} = K'_{CuLn}[CuL_n] + K'_{CuLn}C_{Ln} \quad (4)$$

where $[Cu]_{labile}$, [CuLn], [Ln], K'_{CuLn} and α' are the labile copper concentration, concentration of copper complexed by natural ligand L_n, natural complexing ligand concentration, conditional stability constant of CuL_n complex and the overall side reaction coefficient of Cu^{2+} (excluding the complexation by L_n) which is calculated as before (Campos and van den Berg.

Values of $[Cu]_{labile}$ and $[CuL_n]$ can be expressed as follows:

$$[Cu]_{labile} = i_p/S \quad (5)$$

$$[CuL_n] = [Cu]_T - i_p/S \quad (6)$$

where i_p, S and $[Cu]_T$ represent the maximum peak height at each titration point in Ampere (A), the analytical sensitivity in Ampere/Molar (A/M) and the total copper concentration in Molar (M) at each titration point.

Fig. 5 shows the forward titration curve which represents a plot i_p as Y-axis, equivalent to the concentration of $CuSA_2$, against the total copper concentration $[Cu]_T$ as X-axis for both the (a) UV-digested extract and (b) aqueous extract of Sohag-sample.

It is clear from Fig. 5a, that the relation between the peak current i_p and the total copper $[Cu]_T$ is a straight line. This indicates that Cu is only bound with the added SA ligand to form $CuSA_2$ complex and no other interactions take place with copper. The sensitivity (S) of the titration was found 1.495 A/M, which is calculated from the slope of the titration curve.

Fig. 5b shows the same titration but for the aqueous extract without digestion. The same plot gives another shape consisting of two regions, the first region is a curve that starts at 0.494 μM and ends at 0.504 μM of $[Cu]_T$. The second region is a straight line starts from 0.504 μM of $[Cu]_T$ and have a sensitivity (S = 1.498 A/M), obtained from the slope at the higher end of the titration points. The values of S of the two titrations are so close with a relative error of 0.002. It is obviously clear that the first region in Fig. 5b is related to the equilibrium (competition) that takes place between the natural ligands (L_n) present in the aqueous extract and the added competing ligand (SA). In this region, the added copper is distributed over the natural, L_n, and the added competing ligand, SA. As soon as equilibrium is established, all the added copper is complexed by only SA, resulting in a straight line between the (i_p) and the added copper.

This forward titration of *E.C* extract in the presence of 0.98 mM SA is evaluated using two linearization techniques: (i) van den Berg (1985) method, and (ii) Scatchard (1949) method represented in Eqs. (3) and (4), consequently.

van den Berg linearization is constructed by plotting $[Cu]_{labile}/[CuL_n]$ as ordinates and $[Cu]_{labile}$ as abscissa. Values of C_{Ln} and K'_{CuLn} are determined from the slope and intercept, respectively. Whereas, Scatchard linearization is constructed by plotting $[CuL_n]/[Cu]_{labile}$ as ordinates and $[CuL_n]$ as abscissa. Values of C_{Ln} and K'_{CuLn} are determined in the order from the intercept and slope. Fig. 6a and b correspond to van den Berg and Scatchard transformations, respectively. The two figures reveal that there is only one class of copper complexation, CuL1, where a typical straight line is obtained in both transformations without any curvature.

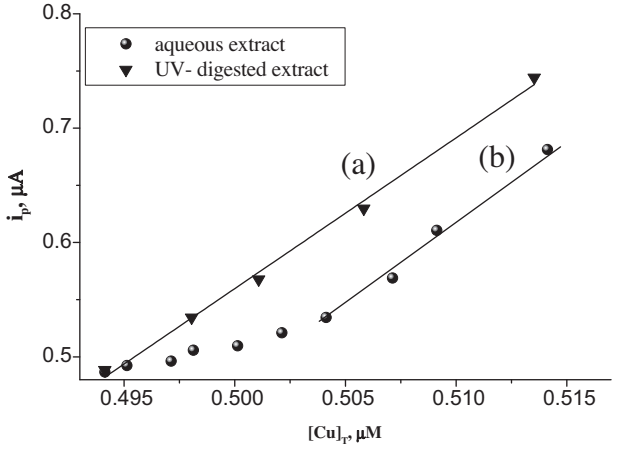

Figure 5 Forward titration; a plot of $[Cu]_T$ *vs.* i_p for: (a) *E.C* aqueous extract, and (b) UV-digested extract.

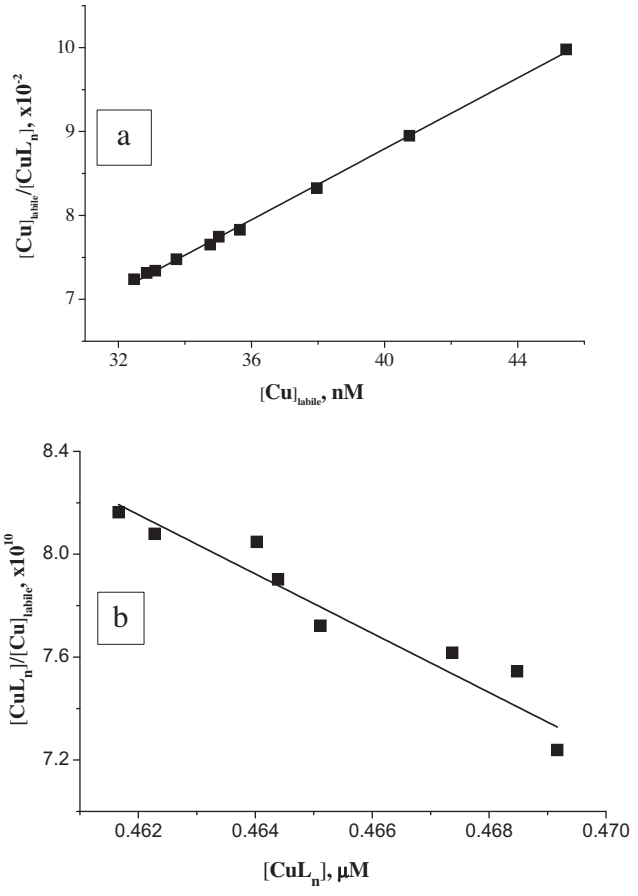

Figure 6 Linearization plot of the data obtained from the forward titration using; (a) van den Berg and (b) Scatchard.

The values of C_{L1} and $\log K'_{CuL1}$, obtained from the two transformations of the forward titration are shown in Table 1. Obviously, The values of $\log K'_{CuL_1}$ and C_{L1} are similar in the two linearization techniques, suggesting that only one class of copper complexation (CuL1) exist with average $\log K'_{CuL1}$ of 18.31 and average C_{L1} of 502 nM. In addition, the average C_{L1} (502 nM) is so close to the $[Cu]_T$ (480 nM) in the extract of the same sample.

3.4.3. Comparison between the forward and reverse titrations

Xue and Sigg (2002) and Ploeger et al. (2005) studied the copper speciation in freshwater samples by the same CLE technique. They concluded that there is no existence of natural ligands capable of making complexes with Cu of $\log K'_{CuLn} > 19.0$. In contrast, Wang and Chakrabarti (2008) studied the copper speciation in Rideau Canal, Canada. They indicated that Cu can form very strong complexes (average $\log K'_{CuLn} \sim 20$ and $C_{Ln} > 100$ nM) with natural ligands found in the Rideau Canal (Wang and Chakrabarti, 2008). The results in Tables 1 and 2 for both *E.C* samples reveal that the first conclusion, by Xue and Sigg (2002) and Ploeger et al. (2005), is more acceptable than the second one where all the values of $\log K'_{CuL1}$ do not exceed 19.0. So it can be concluded that Cu is capable of forming strong complexes with the natural ligands in the aqueous extract of both *E.C* samples.

Our results indicate that the forward titration is capable of determining only the stronger complexation of copper in the *E.C* extract, while the reverse titration is more sensitive to detect the weak as well as stronger complexes of copper. The detection window, in the reverse titration, varied from low to high by changing the concentration of the added SA to detect as many as possible of the natural complexation of Cu. Therefore, the ligands detected by the reverse titration were not totally apparent in the usual forward titrations with copper.

It is important to mention that using AdCSV/CLE with SA as a competing ligand has many advantages, from which: (i) the competing ligand (SA) is characterized by high complexing stability which facilitate the detection of different classes of Cu complexes, (ii) Measurements with AdCSV/CLE technique provide an indispensable, easy, accurate and fast tool for the investigation of the copper complexes in aqueous *E.C* extract.

4. Conclusion

Finally, it can be concluded from our study that; (i) the reverse titration is more sensitive than the forward titration to detect the weak as well as stronger complexes of copper, (ii) there are two types of Cu complexation in the *E.C* extract of the two samples, the first class (L_1) is predominant of very strong complexation with Cu, and the second class (L_2) is minor in the extract and with a much weaker stability constant, (iii) the complexing ligands released from *E.C* after extraction process include different types of compounds (e.g. proteins, amino acids, etc), (iv) the values of stability constants in the present study reflect the high affinity of the natural ligand towards Cu(II) ions, (v) there are some differences among the values of speciation parameters of Sohag- and Mansura-samples as a result of the difference in the habitat of the plant and (vi) the presence of *E.C* in waters as alive or dead material helps eliminating the toxicity of Cu ions by transforming them into less toxic forms through complexation.

References

Apte, S.C., Gardener, M.J., Ravenscroft, J.E., Turrell, J.A., 1990. Examination of copper complexation in natural waters using a combination of cathodic stripping voltammetry and computer simulation. Anal. Chem. Acta. 235, 287–299.

Campos, M.L.A.M., van den Berg, C.M.G., 1994. Determination of copper complexation in sea water by cathodic stripping voltammetry and ligand competition with salicylaldoxime. Anal. Chim. Acta. 284, 481–496.

Charudattan, R. 2001. Are we on top of aquatic weeds? Weed problems, control options, and challenges. International symposium on the World's Worst Weeds. British Crop Protection Council, Brighton, United Kingdom. November 12, 2001.

Coale, K.H., Bruland, K.W., 1988. Copper complexation in the Northeast Pacific. Limnol. Oceanog. 33, 1084–1101.

Crea, P., Stefano, C.D., Milea, D., Sammartano, S., 2008. Formation and stability of mixed Mg^{2+}/Ca^{2+}/phytate species in synthetic seawater media: consequences on ligand speciation. Mar. Chem. 112, 142–148.

Donat, J.R., Lao, K.A., Bruland, K.W., 1994. Speciation of dissolved copper and nickel in South San Francisco Bay: a multi-method approach. Anal. Chim. Acta. 284, 547–571.

Jin, L., Gogan, J., 2000. Copper complexing capacities of freshwaters by adsorptive cathodic stripping voltammetry. Anal. Chim. Acta. 412, 77–88.

Komy, Z.R., 1993. Determination of trace metals in Nile River and ground water by differential pulse stripping voltammetry. Mikrochim. Acta. 111, 239–249.

Laglera, L.M., van den Berg, C.M.G., 2006. Photochemical oxidation of thiols and copper complexing ligands in estuarine waters. Mar. Chem. 101, 130–140.

Leal, M.F.C., Vasconcelos, M.T.S.D., van den Berg, C.M.G., 1999. Copper induced release of complexing ligands similar to thiols by Emiliania huxleyi in seawater cultures. Limnol. Oceanog. 44, 1750–1762.

Masami, G.O., Usui, I., Urano, N., 2008. Ethanol production from the water hyacinth *Eichhornia crassipes* by yeast isolated from various hydrospheres. Afr. J. Microbiol. Res. 2, 110–113.

Moffett, J.W., Brand, L.E., Croot, P.L., Barbeau, K.A., 1997. Cu Speciation and cyanobacterial distribution in harbors subject to anthropogenic Cu inputs. Limnol. Oceanogr. 42, 789–799.

Morel, F.M.M., Hering, J.G., 1993. Principles and Applications of Aquatic Chemistry. John Wiley and Sons, New York (Chapter 6).

Nagai, T., Imai, A., Matsushige, K., Yokoi, K., Fukushima, T., 2007. Dissolved iron and its speciation in a shallow eutrophic lake and its inflowing rivers. Water Res. 41, 775–784.

Ndungu, K.Ndungu, Hurst, M.P., Bruland, K.W., 2005. Comparison of copper speciation in estuarine water measured using analytical voltammetry and supported liquid membrane techniques. Environ. Sci. Technol. 39, 3166–3175.

Nuester, J., van den Berg, C.M.G., 2005. Determination of metal speciation by reverse titrations. Anal. Chem. 77, 11–19.

Ploeger, A., Fischer, E., Nirmaier, H.-P., Laglera, L.M., Monticelli, D., van den Berg, C.M.G., 2005. Lead and copper speciation in remote mountain lakes. Limnol. Oceanog. 50, 995–1010.

Santos-Echeandía, J., Laglera, L.M., Prego, R., van den Berg, C.M.G., 2008. Copper speciation in estuarine waters by forward and reverse titrations. Mar. Chem. 108, 148–158.

Scatchard, G., 1949. The attractions ofproteins for small molecules and ions. Ann. N.Y. Acad. Sci. 7, 660–672.

Schneider, I.A.H., Rubio, J., Misra, M., Smith, R.W., 1995. Stems and roots of eichhornia crassipes as biosorbents for heavy metal ions. Miner. Eng. 8, 979–988.

Templeton, D.M., Ariese, F., Cornelis, R., Danielsson, L.-G., Muntau, H., van Leeuwen, H.P., Lobinski, R., 2000. IUPAC guidelines

for terms related to speciation of trace elements. Pure Appl. Chem. 72, 1453–1470.

Town, R.M., van Leeuwen, H.P., 2005. Measuring marine iron(iii) complexes by CLE-AdSV. Environ. Chem. 2, 80–84.

van den Berg, C.M.G., 1985. Determination of the zinc complexing capacity in seawater by cathodic stripping voltammetry of zinc-APDC complex ions. Mar. Chem. 15, 1–18.

Villamagna, A.M., Murphy, B.R., 2010. Ecological and socio-economic impacts of invasive water hyacinth (*Eichhornia crassipes*): a review. Freshwater Biol. 55, 282–298.

Wang, R., Chakrabarti, C.L., 2008. Copper speciation by competing ligand exchange method using differential pulse anodic stripping voltammetry with ethylenediaminetetraacetic acid (EDTA) as competing ligand. Anal. Chim. Acta. 614, 153–160.

Xue, H.-B., Sigg, L., 2002. A review of competitive ligand-exchange voltammetric methods for speciation of trace metals in freshwater. In: Taillefert, M., Rozan, T.F., (Eds.), Environmental Electrochemistry. Analyses of Trace Element Biogeochemistry, ACS Symposium Series No. 811; American Chemical Society, Washington, DC, pp 336–370.

Xue, H.-B., Sunda, W.G., 1997. Comparison of $[Cu^{2+}]$ measurements in lake water determined by ligand exchange and cathodic stripping voltammetry and by ion-selective electrode. Environ. Sci. Technol. 31, 1902–1907.

Zhang, H., Davison, W., 1995. Performance characteristics of diffusion gradients in thin films for the in situ measurement of trace metals in aqueous solution. Anal. Chem. 67, 3391–3400.